

SCIENTIFIC OPINION

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Safety of proline-specific oligopeptidase as a novel food pursuant to Regulation (EC) No 258/97

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Abstract

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on proline-specific oligopeptidase (Tolerase[®] G) as a novel food ingredient submitted pursuant to Regulation (EC) No 258/97 of the European Parliament and of the Council, taking into account the comments and objections of a scientific nature raised by Member States. The novel food is an enzyme preparation of prolyl-oligopeptidase produced with a genetically modified *Aspergillus niger* self clone strain. The target population is the general adult population. The results from a bacterial reverse mutation test and of an *in vitro* chromosome aberration test did not indicate genotoxicity. The Panel considers that the reported effects observed in a 90-day rat study are treatment-related effects and can be attributed to the higher energy consumption by these animals. Taking into account the intended maximum use level for Tolerase[®] G, its daily consumption would correspond to 2,746 mg TOS/person or to 39.2 mg TOS/kg body weight (bw) per day, when considering a default body weight of 70 kg for an adult person. The margin between this value and the dose in the rats, which caused effects attributable to the excess energy intake, is approximately 45. Noting this margin, the Panel considers that it is unlikely that such effects would occur in human at the intended use levels. The Panel concludes that the NF, Tolerase[®] G, is safe for the intended use at the intended use level.

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Keywords: novel food, proline-specific oligopeptidase, Tolerase[®] G, ingredient, safety

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Summary

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on proline-specific oligopeptidase (Tolerase® G) as a novel food (NF) ingredient submitted pursuant to Regulation (EC) No 258/97¹ of the European Parliament and of the Council, taking into account the comments and objections of a scientific nature raised by Member States. The assessment follows the methodology set in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of NFs and NF ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council. The assessment is based on the data supplied in the original application, the initial assessment by the competent authority of France, the concerns and objections of the other Member States and the responses of the applicant.

The NF is an enzyme preparation of prolyl-oligopeptidase produced with a genetically modified *Aspergillus niger* self clone strain. The composition of this enzyme preparation and its production process including a submerged fermentation do not raise safety concern. The information provided on the genetic modification carried out to obtain the producer strain GEP-44, on the genetic stability of the production microorganism, the expression of the recombinant protein and on the absence of recombinant DNA in the NF, do not raise safety concerns.

The target population is the general adult population. The NF is intended to be consumed just before, during or directly after a meal. The applicant recommends an intake of 40 PPU Tolerase® G per meal (= 0.916 g enzyme preparation) and daily maximum intake of 120 PPU (2x10E6 PPI) Tolerase® G (approximately 2.7 g enzyme preparation) which would correspond to three intakes (three meals) per day.

The results from a bacterial reverse mutation test and of an *in vitro* chromosome aberration test did not indicate genotoxicity.

In a subchronic oral toxicity study carried out in accordance with OECD Guideline 408 and in compliance with Good Laboratory Practice (GLP), the enzyme preparation was administered by gavage to groups with 10 male and 10 female Wistar rats each over a 90-day period in doses of 0 (control group), 2,000, 7,000 and 20,000 mg/kg body weight (bw) per day, corresponding to 518, 1,813 and 5,180 mg dry weight/kg bw per day. The control group received double distilled water. A significantly higher bilirubin level (+16.7%) was seen at the highest dose in males, significantly increased glucose levels were seen in the mid- and high-dose group of females and increased cholesterol level was seen in the high-dose females. The Panel considers that the effects on glucose (+16.5%) and cholesterol (+33%) may be related to the higher calorie intake by female rats. A significant increase in relative weight (+6.4%) of the liver was seen in the high-dose males. For female rats at the highest dose level, a significant increase was seen for both the absolute and relative liver weight (8.4%). The Panel notes that the increase in relative liver weight was the only consistent finding among both sex groups. In both sexes, the relative liver weight of the mid-dose animals was increased, albeit not statistically significant. No treatment-related differences between groups were seen in gross- and histopathology including the liver and the tissues of the gastrointestinal tract. According to the data provided in the study report, the three groups of female rats receiving 518, 1,813 and 5,180 mg dry weight of the test material/kg bw per day did not consume less feed than the control females, resulting to an energy intake by the females of the high-dose group which was about 9.2% higher than in the control group. While the mid-dose group in male rats also did not reduce feed intake, high-dose male had a significantly decreased feed consumption. Upon request of European Food Safety Authority (EFSA), the applicant calculated and compared the total energy intake from the feed and from the test material between the high-dose and control male rats for the 13 time points when feed intake and body weight were recorded. According to these calculations, the total energy intake of high-dose male rats was approximately 3% higher than of the control males.

The Panel considers that the reported effects of significantly higher body weight and body weight gain by high-dose female rats, absolute increased liver weight by high-dose female and relative increased liver weight of high-dose female and male rats, and the significant increase in glucose in mid-dose female rats, are treatment-related effects and can be attributed to the higher energy consumption by these animals. In the absence of differences in liver enzyme plasma levels and the absence of findings in the histopathology of the liver, the Panel considers that the increases of plasma

¹ Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients (OJ L 43, 14.2.1997, p. 1). <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1997R0258:20090120:EN:PDF>

glucose and cholesterol observed in high-dose female rats, and the increase in bilirubin in high-dose males, are not of toxicological concern.

Taking into account the intended maximum use level for Tolerase® G, its daily consumption would correspond to 2,746 mg TOS/person or to 39.2 mg TOS/kg bw per day, when considering a default body weight of 70 kg for an adult person. The margin between this value and the dose in the rats, which caused effects attributable to the excess energy intake, is approximately 45. Noting this margin, the Panel considers that it is unlikely that such effects would occur in human at the intended use levels.

The Panel concludes that the NF, Tolerase® G, is safe for the intended use at the intended use level.

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1. Introduction

1.1. Background and Terms of Reference as provided by the European Commission

On 13 June 2012, the company DSM Food Specialties submitted a request under Article 4 of the Novel Food Regulation (EC) No 258/97² to place on the market a proline-specific oligopeptidase derived from *Aspergillus niger* as a novel food (NF) ingredient.

On 31 July 2014, the competent authority of France forwarded to the Commission its initial assessment report, which came to the conclusion that enzyme preparation of prolyl oligopeptidase produced with a genetically modified *Aspergillus niger* strain meets the criteria for acceptance of a novel food defined in Article (3)1 of Regulation (EC) No 258/97.

On 11 November 2014, the Commission forwarded the initial assessment report to the other Member States. Several Member States submitted comments or raised objections.

The concerns of a scientific nature raised by the Member States can be summarised as follows:

- It is not clear from information provided in the application whether the laboratory concerned is accredited in accordance with an internationally recognised system to carry out the various analyses. In general, it should be noted that only a testing laboratory accredited for the testing spectrum in question should be used for analyses in the context of the authorisation or determination of the substantial equivalence of a novel food.
- The application contains no data on the total protein content or on the purity of the specific prolyl oligopeptidase enzyme.
- The applicant is requested to take into account the potential of *A. niger* to produce mycotoxins in the production's HACCP plan and as part of the in-house control practices.
- As it may be assumed that Tolerase® G does not achieve its spectrum of activity until it reaches the small intestine, it needs to be encased in a special capsule in order to be effective. Safety and tolerance tests should therefore be carried out in such a way that they reflect the galenic formulation of the end product.
- The Member States expressed concerns and requested clarity regarding the target population.
- The consumption of the product in question should not exceed the acceptable intake levels determined on the basis of the toxicological study.
- Concerns were expressed about the *in vivo* efficacy of the product and the potential for indirect undesirable effects on the dietary compliance of subjects suffering from coeliac disease.
- Measurement of the degradation rate of the 'unwanted' substance gluten via a simulated gastric fluid test.
- Concerns were expressed with regard to the consumption of the NF by children.
- Concerns were expressed that the NF may also digest collagen and elastin, which have substantial proportions of proline.
- *Aspergillus niger* can cause invasive disease, particularly in immunocompromised individuals and those with leaky guts. It considered colonisation of the human intestine by the strain of *A. niger* producing the NF, as stated by the applicant is uncertain. Consumption of mycotoxins, e.g. ochratoxins, can cause disease, such as nephrotoxicity, or can be fatal if consumed by an immunocompromised patient population.
- Safety and long-term tolerability of the NF can only be tested in the human context, as there may be specific mechanisms in the human gut and with a human diet that cannot be tested in animal models easily.

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002³, the European Food Safety Authority (EFSA) is asked to carry out the additional assessment for proline-specific oligopeptidase as a novel food in the context of Regulation (EC) No 258/97.

EFSA is asked to carry out the additional assessment and to consider the elements of a scientific nature in the comments raised by the other Member States.

² Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. OJ L 43, 14.2.1997, p. 1–6.

³ Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

2. Data and methodologies

2.1. Data

The assessment of the safety of this NF is based on data supplied in the original application, the initial assessment by the competent authority of France, the concerns and objections of the other Member States and the responses of the applicant (See 'Documentation provided to EFSA'). In accordance with Commission Recommendation 97/618/EC,⁴ the novel food was allocated by the French Authority to Class 5.2, i.e. genetically modified (GM) microorganisms and their products, the host microorganism used for the genetic modification has no history of use as food or as a source of food in the Community under comparable conditions of preparation and intake, i.e. structured schemes I, II, III, IV, V, VI, VII, XIII, IX, XI, XII and XIII of Commission Recommendation 97/618/EC. In the current opinion, these structured schemes are listed in Sections 3.1–3.9.

According to the applicant, the novel food called Tolerase® G 'is intended to be used as ingredient for food supplements, supporting the degradation of proteins. Since Tolerase® G is a proline-specific oligopeptidase, its main activity is exerted on proline-rich proteins, such as gluten, although other proteins besides gluten are also degraded. Tolerase® G is intended, for example, to be consumed alongside (potentially) gluten containing food items, to aid in the degradation of gluten in the gastrointestinal tract, notably in the stomach'. The target population is the general adult population. This assessment concerns only risks that might be associated with consumption, and is not an assessment of the efficacy of the NF with regard to any claimed benefit.

2.2. Methodologies

The assessment follows the methodology set out in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council.

Considering the nature of this novel food, an enzyme produced by a GM *Aspergillus* species, part of the assessment (concerning the genetically modified microorganism) follows also the EFSA Guidance on the submission of a dossier on Food Enzymes (EFSA, 2009a) and the EFSA Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (2006).

3. Assessment

3.1. Specification of the NF

The NF (Tolerase® G) is a prolyl-oligopeptidase produced with a GM *A. niger* self clone strain. The production process includes a submerged fermentation followed by recovery and concentration steps (downstream processing). The final enzyme preparation is a dried granulate obtained after adding up to 30% maltodextrin. The NF is a white-grey to cream-coloured microgranule with a maximum of 5% of the particles being smaller than 63 µm.

The prolyl-oligopeptidase (Enzyme Commission number: EC 3.4.21.26; CAS number: 72162-84-6) is a glycosylated protein (526 amino acids) with a molecular weight of 66 kDa.

This protease specifically cleaves peptide bonds at the carboxylic site of proline residues and releases smaller peptides. The activity of the enzyme can be expressed as PPI (protease picomole international) or PPU (prolyl peptidase units or proline protease units). The assay is based on the release of *p*-nitroanilide (pNA) from the synthetic substrate Z-Gly-Pro-pNA (carbobenzoxy-glycyl-proline-*p*-nitroanilide) at pH 4.6 and 37°C. One PPI is defined as the amount of enzyme required to release one picomole of pNA from Z-Gly-Pro-pNA in 1 s under the conditions of the assay. One PPU is defined as the amount of enzyme required to release one micromole of *p*-nitroanilide (pNA) from Z-Gly-pro-pNA in 1 min under conditions of the assay (pH 4.6, 37.0°C). One PPU therefore amounts to 16,667 PPI.

⁴ Commission Recommendation 97/618/EC: Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council. OJ L 253, 16.9.1997, p. 1–36.

The temperature optimum of the enzyme is around 50°C. The enzyme is stable for 10 h at 50°C and pH 5 with approximately 90% residual activity. The enzyme is inactivated after 5 min at 99°C.

At 37°C, it exhibits maximum activity at pH 4.6 and has approximately 60% of its activity at pH 5.5. At pH values of 7.0 and higher, the enzyme is essentially inactive.

Information on the chemical composition of the prolyl-oligopeptidase (ultrafiltration concentrate) has been provided for four batches; one of them has been used for the toxicological studies (Table 1). The total organic solids (TOS) content is a calculated value derived as 100% minus % water minus % ash. It reflects the organic material isolated together with the target enzyme from the fermentation broth. Further information on the composition of these total organic solids is not available.

Table 1: Chemical compositions provided for four batches of the prolyl-oligopeptidase enzyme (ultrafiltration concentrate)

	Batch 812023201	Batch 8144031001	Batch 814033701	Batch^(a) JLL 03 006 IDF
Ash (%)	0.25	0.50	0.50	0.41
Water (%)	78.9	72.3	72.5	74.6
TOS (%)^(b)	20.9	27.2	27.0	25.0
Protein (%)	14.4	18.9	18.6	17.3

(a): Batch used for the toxicological studies.

(b): The total organic solids (TOS) content is a calculated value derived as 100% minus % water minus % ash.

The enzyme activity of the final enzyme preparation is being standardised by adding up to 30% of maltodextrin. According to the applicant, the enzyme activity of the resulting Tolerase® G granulate is standardised to a level of at least 580,000 PPI/g.

Specifications for Tolerase® G based on the information provided by the applicant are shown in Table 2.

Table 2: Specifications for Tolerase® G

Parameter	DSM Specifications Limits for Tolerase® G	Method
Activity	> 580,000 PPI/g (> 34.8 PPU/g)	Spectrophotometric determination of proline-specific endoprotease activity with <i>N</i> -carbobenzoxy-glycyl-proline <i>p</i> -nitroanilide as a substrate
Appearance	Microgranulate	
Colour	Off-white to orange yellowish. The colour may change from batch-to-batch	
Dry matter	> 94%	Compendia method mentioned in FCC 9 Appendix II
Gluten	< 20 ppm	ELISA kit, COFRAC accredited to ISO 17025
Maltodextrin	≤ 30%	
Sodium benzoate	≤ 0.1%	
Total heavy metals (as lead)	≤ 10 mg/kg	
Lead	≤ 1.0 mg/kg	
Arsenic	≤ 1.0 mg/kg	
Cadmium	≤ 0.5 mg/kg	
Mercury	≤ 0.1 mg/kg	
Microbiology		
Total aerobic plate count	≤ 10 ³ CFU/g	NF EN ISO 4833
Total yeasts and moulds	≤ 10 ² CFU/g	NF V08-059 Nov 2002

Parameter	DSM Specifications Limits for Tolerase® G	Method
Sulfite-reducing anaerobes	≤ 30 CFU/g	NF ISO 15213
Coliforms	(a)	(b)
Enterobacteriaceae	< 10 CFU/g	NF V08-054 April 2009
<i>Salmonella</i>	Absent in 25 g	NF EN ISO 6579 V08-013
<i>Escherichia coli</i>	Absent in 25 g	XP ISO/TS 16649-3 December 2005
<i>Staphylococcus aureus</i>	Absent in 10 g	NF EN ISO 6888
<i>Pseudomonas aeruginosa</i>	Absent in 10 g	DFS-SCL-MIP-W-0105
<i>Listeria monocytogenes</i>	Absent in 25 g	NF EN ISO 16140: 2003
Antimicrobial activity	Absent	JECFA ISSN 1817-7077
Mycotoxins	Absent ^(c)	LC-MS/MS method via SGS in-house method 0033-LC, accredited by the Dutch Accreditation Board (www.rva.nl) under scope L092 item 29

(a): The term 'Coliforms' or 'Coliform Bacteria' refers to a group of lactose fermenting, Gram-negative, facultative anaerobic rods that form acid and

(b): Gas at 35°C in 48 h. This group is used as an indicator for the hygienic status in food industry and quality indicator for water. It is a non-taxonomic group that developed historically. The description of the group has changed over the time and therefore also the genera that are included in it. Initially only the genera of *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter* were included, whereas the group currently includes 15 genera. These genera are all included in the family of Enterobacteriaceae. Therefore, the test for Enterobacteriaceae covers also all the microorganisms found in the test for Coliforms.

(c): Below limits of detection: aflatoxins B1, B2, G1, G2 (< 0.25 µg/kg), total aflatoxins (< 2.0 µg/kg), ochratoxin A (< 0.20 µg/kg), T-2 Toxin (< 5 µg/kg), zearalenone (< 2.5 µg/kg), fumonisins B1 and B2 (< 2.5 µg/kg).

The Panel considers that the information provided on the composition, the batch-to-batch variability and the specifications of the NF is sufficient and does not raise safety concerns.

3.1.1. Stability of the NF

If stored below 15°C, the product shows a loss of activity of less than 5% over a 12-month period and is therefore sufficiently stable.

The Panel considers that the data provided sufficient information with respect to the stability of the NF.

3.2. Effect of the production process applied to the NF

The Tolerase® G enzyme preparation is produced by a controlled submerged fermentation of a selected, pure culture of *Aspergillus niger* GEP-44.

The enzyme is manufactured according to the EU Food Hygiene Regulation (EC) No 853/2004. Pursuant to these regulations, Tolerase® G is manufactured in accordance with current Good Manufacturing Practice for Food (cGMP) and the principles of Hazard Analysis of Critical Control Points (HACCP). The manufacturing process is certified according to Food Safety Systems Certification 22000 (FSSC 22000). According to the applicant, compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services.

The production process consists of the fermentation and a subsequent downstream processing, comprising recovery, purification and concentration. In the final enzyme preparation, the enzymatic activity of the prolyl-endopeptidase is standardised by addition of maltodextrin.

In addition to routine control of the raw materials used in the Tolerase® G fermentation and control of the final product, the applicant has specific measures in place with regard to the microbiological purity throughout the process.

At the end of the fermentation, the recombinant production organism is inactivated.

Results of polymerase chain reaction (PCR) analyses based on the detection of the gene encoding the prolyl-endopeptidase have been provided for three batches of the concentrated enzyme to demonstrate the absence of recombinant DNA.

According to the applicant, all raw materials used in the production of the enzyme meet specifications regarding the absence of mycotoxins. Raw materials used in the fermentation or formulation steps are either tested by the applicant or confirmation on compliance with respective EU regulations is requested from the suppliers.

A. niger is known to produce ochratoxin A and fumonisins (Blumenthal, 2004; Palencia et al., 2010; Frisvad et al., 2011). Consequently, these are routinely analysed together with a broader spectrum of other mycotoxins by the applicant in the NF. According to the specifications, they must be absent, i.e. below the limits of detection of the applied analytical method (Table 2). *A. niger* has been reported to produce other secondary metabolites such as nigragilline, nigerazine B and malformins (Blumenthal, 2004).

Quantitative data on the contents of these metabolites have not been provided in the application. In a screening of industrial *A. niger* strains, malformins were detectable via tandem mass spectrometry but were not quantified because of lack of sufficient amounts of pure authentic standards (Frisvad et al., 2011).

The Panel considers that the production process is sufficiently described and does not raise concerns about the safety of the NF.

3.3. History of the organism used as a source of the NF

A. niger is a fungus belonging to the phylum Ascomycota which has been used for many years in the food and pharmaceuticals industry to produce enzymes and chemical substances (such as citric acid).

In order to assess the safety of microorganisms (and viruses) for use in food and feed, EFSA has developed the concept of a Qualified Presumption of Safety (QPS) (EFSA, 2007). According to EFSA's current opinion on the list of biological agents to which the QPS assessment method may be applied, it does not recommend including *A. niger* in the QPS list, since some strains may produce the mycotoxins (EFSA BIOHAZ Panel, 2013).

The NF was tested negative for the presence of mycotoxins at the limits of detection provided in Section 1 (specifications).

Genetic Modification:

Regarding the following Sections 3.4–3.8 (GMM): EFSA Enzyme Guidance refers to EFSA GMM Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (2011). This NF would fall under Category 2: Complex products in which both GMMs and newly introduced genes are no longer present (e.g. cell extracts, most enzyme preparations). For such applications, Chapter III, Sections B.2.2. and B.4.1. of this guidance apply.

3.4. Effect of the genetic modification on the properties of the host organism

The NF is produced by submerged fermentation of the *Aspergillus niger* strain GEP-44 stably expressing the *gepA* gene of *A. niger*. To generate the producer strain GEP-44, the *gepA* gene from *Aspergillus niger* G-306 was inserted into predefined loci of the genome of the recipient *Aspergillus niger* strain ISO 508 (which is derived from *Aspergillus niger* GAM53 (DS3045)) and then further selected for multiple copies of the *gepA* gene via spontaneous recombination events. The production strain GEP-44 contains 20 copies of the *gepA* gene.

The production strain GEP-44 is derived from the DSM's GAM-ISO strain lineage of *A. niger* strains (van Dijck et al., 2003). Both the parental strain GAM53 (DS3045) and the recipient strain (ISO 508) were approved as suitable host strains for the construction of genetically modified organisms (GMOs) belonging to Group I safe microorganisms and as a GMO obtained through selfcloning⁵ by the Dutch

⁵ Self-cloning as defined in the European Directive on the Contained Use of Genetically Modified Microorganisms 98/81/EC (revision of 90/219/EC): Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent) with or without prior enzymatic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting microorganism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular microorganisms.

Authorities (1994). Similarly, the recombinant prolyl-oligopeptidase producer strain GEP-44 has been classified by the Dutch authorities as well as the French Genetic Committee as Group I safe microorganisms and as a GMO self-clone (CGG, 2003; Dutch Authorities, 2003). On this basis, the strain is approved for large scale production of prolyl-oligopeptidase.

In response to EFSA request to provide detailed information about the genetic modifications carried out to obtain the producer strain GEP-44 following the EFSA Guidance on the risk assessment of genetically modified microorganisms (GMMs) and their products intended for food and feed use (2011), the applicant provided information of the characteristic of the vectors used, the history of previous and further genetic modifications to obtain the producer strain GEP-44 and the verification of the sequences actually inserted into the chromosome and the copy number of the inserts by Southern analysis. According to the additional data provided, the Panel considers that the genetic modifications do not raise safety concerns.

On the basis of the provided information the Panel concludes that the recombinant production strain should not be less safe than the original GAM-53 (DS3045) host strain.

3.5. Genetic stability of the genetically modified microorganism (GMM)

The strains belong to the *Aspergillus niger* GAM-lineage – from which both the host ISO 508 and the recombinant prolyl-oligopeptidase production strain GEP-44 were derived. According to the applicant, the whole GAM-lineage is stored since the 1970s at their laboratory. New cultures are frequently derived from stock material and tested after many generations on morphological, growth, production and product characteristics. According to the applicant, these characteristics remain stable except that after plating out, a low frequency of morphologically dissimilar colonies are found. This is, however, considered a normal phenomenon observed for the parental as well as the highly selected industrial strains. The stability of the ISO strain and the prolyl-oligopeptidase production strain does not differ from the parental GAM strains.

In response to EFSA's request to provide data on the stability of the specific strain (GEP-44) that is the subject of the present application, the applicant provided data on both the phenotypic and genetic stability. The phenotypic stability of the strain was established by measuring the enzymatic activity of the prolyl-oligopeptidase in three independent batches of the food enzyme, showing no substantial differences. The Panel considers that the genetic stability was confirmed by comparing the genotype of the producer strain GEP-44 at the end of the fermentation with the strain of the original working cell bank that was used as inoculum for the fermentation and with the recipient strain ISO 508 using PCR analysis of two key genetic makers related to the enzyme production.

3.6. Specificity of expression of novel genetic material

The expression vectors were sequenced and the sequence of the prolyl-oligopeptidase was confirmed. Data of the sequences were also provided upon EFSA request. The size of the protein product was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and was found to be at the expected value. Activity measurements showed that the enzyme catalysed the expected enzymatic reaction.

3.7. Transfer of genetic material from the GMM

The inserted *gpa* gene encoding the prolyl-oligopeptidase is exactly the same as that present in *A. niger* by nature. Since the prolyl-oligopeptidase expression unit is integrated into the chromosome and the genetic material inserted does not contain an *Escherichia coli* origin of replication or any other *E. coli* sequences, the Panel considers that it is unlikely that this genetic material can be transferred from the *A. niger* production organism to another, non-related, organism.

The production strain has been recognised both by the French as well as by the Dutch competent authorities as a strain obtained by self-cloning (CGG, 2003; Dutch Authorities, 2003). Apart from the overexpression of this gene, no other genes are expressed other than the genes naturally present in the genome of the recipient strain of *A. niger*.

Furthermore, three batches of the food enzyme were analysed to ensure the absence of residual recombinant DNA using primers targeting the *gpa* gene. The results of the analyses demonstrate that no recombinant DNA is present in the enzyme preparation.

3.8. Ability of the GMM to survive in the human gut

Following a request from EFSA to provide information on inactivation of the producer strain after the fermentation process, the applicant informed that the producer strain is killed by incubating the biomass with sodium benzoate (0.4% w/w) during up to 6 h at pH 4.0 and 30°C). According to the applicant, the inactivation efficiency is routinely monitored by plating samples in duplicate on Plate Count Agar plates. Tests results of three batches (EDP812012601, DP812018501 and EDP812002101) did not find colony-forming units of *A. niger*.

The host organism is effectively inactivated after the fermentation process and no living host cells are present in the final product. Therefore, the Panel considers that colonisation of the human gut with prolyl-oligopeptidase overexpressing *A. niger* cells cannot occur.

3.9. Anticipated intake/extent of use of the NF

The intended target population is the adult general population.

According to the applicant, the product is intended to be consumed just before, during or directly after a meal. The applicant recommends an intake of 40 PPU Tolerase® G per meal (= 0.916 g enzyme preparation) and a daily maximum intake of 120 PPU (2x10E6 PPI) Tolerase® G (= 2.7 g enzyme preparation) which would correspond to three intakes (three meals) per day.

3.10. Information from previous exposure to the NF or its source

The NF has no history of food consumption. Various strains of *A. niger* have been used not only for food production, particularly for food enzymes (e.g. α -amylase and hemicellulase), but also for the production of citric and gluconic acid, for glucosamine hydrochloride (EFSA 2009b).

The safety of prolyl-oligopeptidase contained in the Tolerase® G preparation and produced by the *Aspergillus niger* CEP-44 has been previously assessed by AFSSA (2005) as a food processing aid in beer brewing and is approved for this use in France and Denmark (Foedevarestyrelsen, 2006).

3.11. Nutritional information on the NF

Taking into account the composition of the NF and the intended daily intake levels (2.7 g; approximately 11 kcal), the Panel considers that the consumption of the NF is not nutritionally disadvantageous.

3.12. Microbiological information on the NF

The Panel considers that the microbiological information provided does not raise safety concerns.

3.13. Toxicological information on the NF

All toxicological tests were carried out using an enzyme preparation (Batch No JLL 03 006 IDF) with 183,334 PPI (= 11 PPU) per gram and with a TOS value of 25.2% (thus 728,335 PPI or 43.7 PPU per gram of TOS) produced at pilot plant scale. The characteristics were provided in the dossier (Annex 11 of the 'consolidated dossier'). The Panel considers that the test item used for the toxicity tests was representative for the NF.

3.13.1. Absorption, distribution, metabolism and excretion

No information was provided.

3.13.2. Genotoxicity

A bacterial reverse mutation test using *Salmonella* Typhimurium strains TA98, TA100, TA1535 and TA1537 and the *E. coli* strain WP2 uvrA was carried out. In initial tests using the 'plate incorporation method', the enzyme preparation induced in strain TA98 with metabolic activation (S9 mix), and in strains TA1535 and TA1537, without S9 mix an increase in the number of revertants in comparison with the negative control. At the same time, a dense bacterial layer was detected on the plate. The Panel considers that this was attributed to the presence of histidine or protein. In a second test sequence using the 'treat and plate' method, the test material did not induce genotoxicity up to the highest tested dose of 5,000 µg/plate.

In vitro chromosome aberration testing using human lymphocytes was carried out in accordance with OECD Guideline 473. In two separate experiments, no increase in the number of cells with structural chromosome aberrations was observed with or without S9 mix up to the highest tested dose of 5,000 µg/mL.

The Panel considers that the data do not indicate genotoxicity.

3.13.3. Subchronic toxicity studies

In a subchronic oral toxicity study carried out in accordance with OECD Guideline 408 and in compliance with GLP, the enzyme preparation was administered by gavage to groups with 10 male and 10 female Wistar rats each over a 90-day period in doses of 0 (control group), 2,000, 7,000 and 20,000 mg/kg body weight (bw) per day, corresponding to 518, 1,813 and 5,180 mg dry weight/kg bw per day. The control group received double distilled water. Feed (12.2 MJ/kg, 19% protein, 3.3% fat, 4.9% fibre, 6.7% ash) was available *ad libitum*.

All the animals survived the treatment period and regular observation revealed no clinically relevant effects. In the functional observation battery (FOB), no adverse effects were observed. In comparison to the negative control, a significantly lower food intake was seen in high-dose males in the first week (16%) and in the last part of the study (7–11%), and for mid-dose males, in the first week only. No statistically significant difference in body weight was seen between the male groups. In the high-dose females, significantly higher body weight and body weight gain were seen throughout the study. The food intake was not significantly different between the female groups except for a significant decrease seen in the first week for mid- and high-dose females. Both the lower food intake in males and the higher body weight seen in females could be explained by the increased amount of calories received through administration of the test compound.

A statistically significant decrease in thrombocyte counts and a significantly increased mean corpuscular haemoglobin (MCH) were seen in the mid- and high-dose females in comparison to the control group. Erythrocytes were significantly decreased in the high-dose females. The only statistically significant findings in female rats in the mid dose were increased MCH and decreased number of thrombocyte counts. However, the Panel notes that in male rats such an effect was not observed, that there was no clear dose-response and that the mean value for thrombocyte counts in the female control group was two standard deviations (SDs) higher than the mean of historical control data and that the mean value for MCH of the control group was one SD below the mean value of historical control data.

In clinical chemistry, a significantly higher bilirubin level (+16.7%) was seen at the highest dose in males, but not in females. In females, significantly increased glucose levels were seen in the mid- and high-dose group. Significantly increased cholesterol level was seen in the high-dose females. The Panel considers that the effects on glucose (+16.5%) and cholesterol (+33%) may be related to the higher calorie intake by female rats.

A significant increase in relative weight (+6.4%) of the liver was seen in the high-dose males. For female rats at the highest dose level, a significant increase was seen for both the absolute and relative liver weight (8.4%). The Panel notes that the increase in relative liver weight was the only consistent finding among both sex groups. In both sexes, the relative liver weight of the mid-dose animals was increased, albeit not statistically significant.

No treatment-related differences between groups were seen in gross- and histopathology including the liver and the tissues of the gastrointestinal tract.

According to the data provided in the study report, the three groups of female rats receiving 518, 1,813 and 5,180 mg dry weight of the test material/kg bw per day did not consume less feed than the control females, resulting to an energy intake by the females of the high-dose group which was about 9.2% higher than in the control group. While the mid-dose group in male rats also did not reduce feed intake, high-dose males had a significantly decreased feed consumption. Upon request of EFSA, the applicant calculated and compared the total energy intake from the feed and from the test material between the high-dose and control male rats for the 13 time points when feed intake and body weight were recorded. According to these calculations, the total energy intake of high-dose male rats was approximately 3% higher than of the control males.

The Panel considers that the reported effects of significantly higher body weight and body weight gain by high-dose female rats, absolute increased liver weight by high-dose female and relative increased liver weight of high-dose female and male rats, and the significant increase in glucose in mid-dose female rats, are treatment-related effects and can be attributed to the higher energy

consumption by these animals. In the absence of differences in liver enzyme plasma levels and the absence of findings in the histopathology of the liver, the Panel considers that the increases of plasma glucose and cholesterol observed in high-dose female rats, and the increase in bilirubin in high-dose males, are not of toxicological concern.

3.13.4. Human studies

The applicant provided three human studies conducted with prolyl-oligopeptidase derived from *A. niger* and produced by the applicant:

In a human intervention study with a prolyl-oligopeptidase derived from *A. niger* (Tack et al., 2013), 16 diagnosed coeliac patients were fed slices of toast containing a total of 7 g gluten along with the enzyme preparation (168 PPU) in an initial phase (safety phase) lasting 14 days. Following a 14-day washout phase, 14 patients were divided into two groups and given slices of toast containing 7 g gluten combined with the enzyme preparation or a placebo over a further 14-day period (efficacy phase). Two patients who had participated in the first phase were excluded from the second phase on ethical grounds because their coeliac symptoms had worsened. According to the article, the enzyme preparation was well tolerated by the participants during the study, although in the second phase there were no differences between the two groups with regard to the coeliac parameters tested (symptom questionnaire, histological and immune parameters of intestinal mucosal biopsy, serum antibodies and adverse effects). The Panel considers that this study does not provide relevant information for the safety of the novel food.

In a randomised double-blind placebo-controlled cross-over trial, 18 adult gluten-sensitive volunteers received a nasogastric tube and consumed a gluten-free breakfast added with 0.5 g of gluten (as 'hidden gluten') and 166 kPPI, 83 kPPU of Tolerase® G enzyme or placebo with a 1-week washout period in between. In the evening after each test day, subjects were asked to complete a gastrointestinal symptom rating scale (GSRS). The GSRS included 15 items and used a 7-point Likert scale in which 1 represents no symptoms and 7 the highest level of symptoms. A statistical analysis was performed using SPSS statistics 23 on the intention-to-treat population using Wilcoxon signed rank paired test at $\alpha = 0.05$ with a Bonferroni correction to detect significant differences. No significant differences were observed in GSRS scores between the different test conditions (porridge with placebo, low-dose and high-dose Tolerase® G). The Panel notes that only a summary of the unpublished study was provided (DSM, 2016).

In addition a randomised, double-blind, placebo-controlled, cross-over study was performed with 12 healthy volunteers (18–45 years of age) receiving 6.1 mL *Aspergillus niger*-derived prolyl endoprotease with 1.6 Mio PPI (96 PPU) produced by the applicant or placebo (Salden et al., 2015). At least 1-week washout was between two test days when subjects received a low calorie gluten containing meal plus the enzyme preparation or placebo and a high calorie gluten containing meal plus the enzyme preparation or placebo. The meal was administered together with the enzyme preparation or the placebo transnasally through a triple lumen catheter. The two distal lumens were positioned 5 and 15 cm distal to the pylorus to obtain samples of the content from the stomach and the duodenum, respectively. The primary objective was to assess the efficacy on gluten degradation in a low and high calorie meal in healthy subjects. At the end of each test day, study participants were asked to fill a 'Symptoms Diary' questionnaire with eight items related to gastrointestinal symptoms (abdominal discomfort, abdominal pain, abdominal distension, constipation, diarrhoea, flatulence, eructation and nausea) and rated them on a 5-point Likert scale. Other study objectives were to investigate the presence of the *Aspergillus niger*-derived enzyme in the stomach and the duodenum and gastrointestinal symptoms. The article reported that there were no statistically significant differences between gastrointestinal symptoms of the verum and the placebo groups. Since the data were not presented in the published article, the applicant provided the detailed information in response to a request of EFSA, confirming the information provided in the published article. To assess the presence of the enzyme in gastric and duodenal samples, the protein fraction from such samples was put on a SDS-PAGE and subsequently stained with Coomassie Blue. According to the article, the enzyme could be shown on the gel in 14 out of 15 gastric samples, but in no sample obtained from the duodenum. The Panel notes that the staining presented in the article may suggest faint signals at 10 and 15 min after start of the meal infusion, but that there was no signal after 30 min. The Panel considers that the data provided in this study indicate degradation of the enzyme in duodenum and that the consumption of 1.6 Mio PPI of this enzyme did not cause acute gastrointestinal symptoms in healthy subjects.

3.14. Allergenicity

The applicant determined on the basis of bioinformatic methods (sequence homology) using the database allermatch (www.allermatch.org) that prolyl-oligopeptidase derived from *A. niger* does not manifest any sequence identity of greater than 35% with known allergens over a segment of at least 80 amino acids. *In silico* analysis of pepsin digestibility of the protein sequence using the program 'PeptideCutter' (ExPASy) performed by the applicant suggested degradability of the enzyme.

A 2.4% prevalence of sensitivity to *Aspergillus* spp. was reported for children (n = 714) with an age of 13 years in cohort in New Zealand measured by a skin prick test (SPT) and 2% was reported for 450 children and adults in the United Kingdom (Cullinan et al., 1997).

Occupational sensitisation against enzymes produced by *Aspergillus* species and their dust spores have been reported in the literature (Cullinan et al., 1997; Bernstein et al., 1999).

In a study by Bindslev-Jensen et al. (2006), 400 consecutive adult subjects with diagnosed allergy to inhalation allergens, food allergens, bee or wasp were enrolled and were skin prick tested against 19 different commercial enzymes used in the food industry (including three enzymes produced by *A. niger* and seven enzymes produced by *Aspergillus oryzae*). Thirteen patients showing positive results in the SPT were subsequently challenged orally with the respective enzymes in a double-blind, placebo-controlled protocol. Only one reaction to a placebo challenge was seen. No positive challenges to the enzymes positive in SPT.

The Panel considers that the allergenic risk of the novel food is not dissimilar as of other food enzymes produced by *Aspergillus*.

4. Discussion

The NF is an enzyme preparation of prolyl-oligopeptidase produced with a genetically modified *A. niger* strain. The composition of this enzyme preparation and its production process including a submerged fermentation do not raise safety concern. The information provided on the genetic modification carried out to obtain the producer strain GEP-44, on the genetic stability of the production microorganism, the expression of the recombinant protein and on the absence of recombinant DNA in the novel food, do not raise safety concerns.

The target population is the general adult population. The NF is intended to be consumed just before, during or directly after a meal. The applicant recommends an intake of 40 PPU Tolerase® G per meal (= 0.916 g enzyme preparation) and daily maximum intake of 120 PPU (2x10E6 PPI) Tolerase® G (approximately 2.7 g enzyme preparation) which would correspond to three intakes (three meals) per day.

The results from a bacterial reverse mutation test and of an *in vitro* chromosome aberration test did not indicate genotoxicity.

Taking into account the intended maximum use level for Tolerase® G, its daily consumption would correspond to 2,746 mg TOS per person or to 39.2 mg TOS/kg bw per day, when considering a default body weight of 70 kg for adults. The margin between this value and the mid dose in the rats, which caused effects attributable to the excess energy intake, is approximately 45. Noting this margin, the Panel considers that it is unlikely that such effects would occur in human at the intended use levels.

5. Conclusions

The Panel concludes that the NF, Tolerase® G, is safe for the intended use at the intended use level.

Documentation provided to EFSA

- 1) Letter from the European Commission to the European Food Safety Authority with the request for a scientific opinion on the safety of proline-specific oligopeptidase as a novel food ingredient. Ref. Ares(2015)5348394, dated 25/11/2015.
- 2) Dossier 'Application for the approval of Tolerase™ G, a proline-specific oligopeptidase derived from *Aspergillus niger* to be used as a food supplement aiding in the digestion of gluten'. DSM no. 1146-001 received on 3 December 2015.
- 3) Dossier 'Application for the approval of Tolerase® G, a proline specific oligopeptidase (prolyl oligopeptidase) derived from *Aspergillus niger* to be used as an ingredient in food supplements ('Consolidated dossier Feb 2016') received on 19 February 2016.

- 4) On 13 June 2016 and 12 July 2016, EFSA sent requests to the applicant to provide missing/complementary information.
- 5) On 11 July 2016, EFSA received the missing information as submitted by the applicant.
- 6) Initial assessment report carried out by the Food Safety Authority of France: 'Opinion of the National Agency for Food, Environmental and Occupational Health and Safety concerning a request for authorisation to place on the market a novel food or food ingredient: enzyme preparation containing a protease activity.' Avis de l'Anses Saisine n° 2014-SA-0086 Saisines liées n° 2013-SA-0112 et 2012-SA-0120.
- 7) Member States' comments and objections.
- 8) Response by the applicant to the initial assessment report and the Member States' comments and objections.

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Abbreviations

bw	bodyweight
CAS	Chemical Abstracts Service
cGMP	current Good Manufacturing Practice
FOB	functional observation battery
GLP	Good Laboratory Practice
GM	genetically modified
GMM	genetically modified microorganism
GMO	genetically modified organism
GSRS	gastrointestinal symptom rating scale
HACCP	Hazard Analysis of Critical Control Points
ISO	International Organization for Standardization
MCH	mean corpuscular haemoglobin
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
NF	novel food
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
pNA	p-nitroanilide
PPI	protease picomole international
PPU	prolyl peptidase units or proline protease units
QPS	Qualified presumption of safety (QPS)
SD	standard deviation
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SPT	skin prick test
TOS	total organic solids
Z-Gly-Pro-pNA	carbobenzoxy-glycyl-proline- <i>p</i> -nitroanilide